Supplementary Note:

Antibodies:

Antibodies	Clone	Vender	Catlog number	RRID identifiers
Rabbit monoclonal anti-human Itk	clone Y402	Abcam	Cat # ab32507	RRID:AB_2296410
BV711 rat monoclonal anti-mouse CD4	clone RM4-4	BD Biosciences	Cat # 740651	RRID:AB_2740340
BUV805 rat monoclonal anti-mouse CD8	clone 53 6.7	BD Biosciences	Cat # 564920	RRID:AB_2716856
BV786 Armenian monoclonal anti-mouse CD69	clone H1.2F3	BD Biosciences	Cat # 564683	RRID:AB_2738890
APC Armenian monoclonal anti-mouse CD69	clone H1.2F3	BD Biosciences	Cat # 560689	RRID:AB_1727506
PE-Cy7 mouse monoclonal anti-human CD69	clone FN50	BD Biosciences	Cat # 557745	RRID:AB_396851
Mouse monoclonal anti-mouse/human phospho-LAT (Tyr171)	clone I58-1169	BD Biosciences	Cat # 558392	RRID:AB_647174
Biotin rat monoclonal anti-mouse/human CD45R/B220	clone RA3-6B2	BioLegend	Cat # 103204	RRID:AB_312989
Biotin rat monoclonal anti-mouse CD49b, pan-NK cell	clone DX5	BioLegend	Cat # 108904	RRID:AB_313411
Biotin rat monoclonal anti-mouse TER-119/Erythroid Cells	clone TER-119	BioLegend	Cat # 116204	RRID:AB_313705
Biotin rat monoclonal anti-mouse CD24	clone M1/69	BioLegend	Cat # 101804	RRID:AB_312837
Biotin rat monoclonal anti-mouse CD4	clone GK1.5	BioLegend	Cat # 100404	RRID:AB_312689
PE mouse monoclonal anti-	clone OKT3	BioLegend	Cat # 317308	RRID:AB_571913

human CD3e				
Alexa Fluor 488 rat monoclonal anti-mouse IRF4	clone IRF4.3E4	BioLegend	Cat # 646406	RRID:AB_2563267
PE-Cy7 rabbit monoclonal anti-mouse/human phopspho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204)	clone 197G2	Cell Signaling Technology	Cat # 98168S	
Rabbit polyclonal anti-mouse/human phospho-LAT (Tyr191)		Cell Signaling Technology	Cat # 3584S	RRID:AB_2157728
Rabbit polyclonal anti-mouse/human LAT		Cell Signaling Technology	Cat # 9166	RRID:AB_2283298
Mouse monoclonal anti-myc tag	clone 9B11	Cell Signaling Technology	Cat # 2276S	RRID:AB_331783
Anti-mouse IgG, HRP-linked Antibody		Cell Signaling Technology	Cat # 7076	RRID:AB_330924
Anti-rabbit IgG, HRP-linked Antibody		Cell Signaling Technology	Cat # 7074	RRID:AB_2099233
Anti-Phosphotyrosine Antibody	clone 4G10	EMD Millipore or Weiss lab	Cat # 05-321	RRID:AB_309678
Mouse monoclonal anti-alpha tubulin	clone B-5-1-2	Sigma-Aldrich	Cat # T5168	RRID:AB_477579
Rabbit polyclonal anti-mouse/human phospho-LAT (Tyr132)		Thermo Fisher Scientific	Cat # 44-224	RRID:AB_2533608
Biotin rat monoclonal anti-mouse/human CD11b	clone M1/70	Tonbo Biosciences	Cat # 30-0112-U500	RRID:AB_2621639
Biotin armenian hamster monoclonal anti-mouse CD11c	clone N418	Tonbo Biosciences	Cat # 30-0114-U100	RRID:AB_2621640
Biotin rat monoclonal anti-mouse CD8a	clone 53-6.7	Tonbo Biosciences	Cat # 30-0081-U500	RRID:AB_2621638

Biotin rat anti-mouse CD19	clone 1D3	Tonbo Biosciences	Cat # 30-0193-U500	RRID:AB_2621641
Mouse monoclonal anti-mouse/human ZAP-70	clone 1E7	Weiss lab		

Protein expression and purification

ZAP-70 kinase domain

The recombinant ZAP-70 kinase domain, used for *in vitro* phosphorylation assays, was expressed and purified as described previously[1]. The gene encoding the human ZAP-70 kinase domain (residues 327-606), with an N-terminal methionine and C-terminal His_6-tag was encoded in a pFastBac1 vector, which was used for baculovirus-mediated expression of the protein in Sf21 insect cells. The kinase domain was isolated to high purity from these cells by Ni affinity chromatography followed by cation exchange chromatography and size exclusion chromatography. The enzyme was stored at a concentration of roughly 45 μM in a buffer containing 10 mM HEPES, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM reducing agent TCEP (tris(2-carboxyethyl)phosphine), and 10% glycerol.

LAT cytoplasmic region

A wild-type construct bearing the cytoplasmic region of human LAT (residues 30-233, isoform 2 numbering) was previously encoded in a pET-28b vector with an N-terminal His_6-tag and a Tobacco Etch Virus (TEV) protease cleavage site[2]. We produced Y127F and Y132F variants of this construct by site-directed mutagenesis. In a typical purification of these constructs, the protein was expressed from this plasmid in a 2 L culture of BL21 (DE3) cells in terrific broth. Cells were grown to an optical density of approximately 0.5 at 600 nm, after which they were cooled to 18°C, and incubated with 0.5 mM IPTG overnight to induce protein expression. Cells were harvested by centrifugation at 4000 × *g* for 30 min in 1 L bottles, then each pellet was resuspended in 15 mL of a buffer containing 50 mM Tris, pH 8.0, 300 mM NaCl, 5 mM imidazole, 2 mM 2-mercaptoethanol, 10% glycerol, and a cocktail of protease inhibitors.

The cells were lysed using a probe sonicator, and the lysate was clarified by centrifugation at 35,000 × *g* for 45 min. The supernatant from the centrifuged lysate was applied to a 5 mL GE HisTrap column, which was then washed extensively with the aforementioned lysis buffer, followed by an equivalent buffer with only 50 mM NaCl. Next, the protein was eluted from the HisTrap column using a buffer containing 50 mM Tris, pH 8.0, 50 mM NaCl, 250 mM imidazole, 2 mM 2-mercaptoethanol, and 10% glycerol. The eluate was applied directly to a 5 mL GE HiTrap Q anion exchange column. The protein was eluted over a salt gradient from 50 mM NaCl to 1 M NaCl in a Tris buffer at pH 8.0. The desired fractions were pooled, concentrated, and then further purified using an Superdex 200 16/600 gel filtration column in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM TCEP, and 10% glycerol. Note that the His_6-tag was not cleaved off of the LAT protein constructs. The recombinant LAT proteins were stored in the gel filtration buffer at a concentration of approximately 100 μM.

PLCγ1 N-terminal SH2 domain

As with the LAT cytoplasmic domain constructs, this protein (human PLCγ1 residues 547-660) was also cloned into a pET-28b vector with an N-terminal His_6-tag and a TEV protease cleavage site. The protein was expressed and partially purified over HisTrap and HiTrap Q columns as done for the LAT proteins. Then, the His_6-tag was removed by overnight incubation with TEV protease, and the untagged protein was further isolated by subtractive affinity chromatography over another HisTrap column. The SH2 domain was further purified by gel filtration using a Superdex 75 16/600 column in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP. The protein was stored at a concentration of 330 μM.

Tandem PLCγ1 N-terminal SH2 domains fused to eGFP

A construct was prepared in a pET-based vector containing two copies of the human PLCγ1 N-terminal SH2 domain (residues 547-660) separated by 20 residue Gly/Ser linker. This tandem SH2 construct was flanked by a His_6-tag and TEV protease cleavage site at the N-terminus and a C-terminal eGFP tag. This protein was expressed and purified as described above for the isolated PLCγ1 N-terminal SH2 domain.

Isolation of peptides

All peptides used in this study were produced recombinantly in *Escherichia coli* as N-terminal His_6-SUMO fusion proteins using the established protocol described previously[1]. Briefly, after over-expression and enrichment over Ni-NTA resin, the peptides were cleaved from their SUMO tags using the SUMO-specific protease Ulp1. The resulting cleaved peptides were further purified by reverse phase high-performance liquid chromatography (RP-HPLC) to a purity greater than 95%. The identities of the isolated peptides were confirmed by electrospray ionization mass spectrometry (ESI-MS). In all cases, purified peptides were lyophilized and then re-dissolved in a buffer that was compatible with the downstream application.

For measurements of *in vitro* phosphorylation kinetics, three peptides were used, spanning human LAT residues 120-139, either with the native G131, a G131D mutation, or G131E mutation. In each case, Y127 was mutated to phenylalanine so as not to confound kinetic measurements by including two phosphorylation sites. For PLCγ1 N-SH2 binding measurements, the peptides were also derived from residues 120-139 in human LAT and either contained the wild-type sequence or a G131D mutation. For these peptides, Y127 was not mutated.

The phosphorylated peptides that were used for SH2 binding measurements were produced by enzymatic phosphorylation of purified, unphosphorylated peptides using the Syk kinase domain. A typical large-scale phosphorylation reaction was carried out on ~20 mg of pure peptide on a 15 mL scale (~0.5 mM peptide). The reactions were conducted in a buffer containing 10 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2 mM sodium orthovanadate, 5 mM ATP, and 1 μM Syk kinase domain. Reactions were allowed to proceed for 4 h at 25°C and then purified by RP-HPLC. We note that singly- and doubly-phosphorylated peptides were readily separated by RP-HPLC. The doubly-phosphorylated wild-type and G131D peptides were isolated to greater than 95% purity, as determined by RP-HPLC, and their identities and phosphorylation states were confirmed by ESI-MS.

***In vitro* phosphorylation assays**

Immunoblot analysis of LAT tyrosine phosphorylation kinetics

All reactions were performed in a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 2 mM sodium orthovanadate, and 2 mM TCEP. In a typical reaction, LAT and ZAP-70 were diluted into this buffer to final concentrations of 5 μM and 1 μM, respectively. Reactions were initiated by adding ATP from a concentrated stock to a final concentration of 1 mM. Reactions with the wild-type construct and the Y127F and Y132F mutants were carried out in parallel out at 25°C. At various time points, aliquots were removed and mixed with a 2x SDS-PAGE gel loading dye supplemented with 25 mM EDTA. Samples were run on a 12% Tris-glycine SDS-PAGE gel; then, the proteins were transferred to a PVDF membrane using CAPS transfer buffer (10 mM CAPS, pH 11, 10% MeOH) in a semi-dry transfer apparatus. The membrane was blocked for 1 h at 25°C in TBST containing 4% powdered milk (w/v), the primary antibody was applied overnight at 4°C in in TBST containing 4% powdered milk (w/v) (anti-p-Y132, 1:5000 dilution, 4G10, 1:2,000 dilution). After extensive washing with TBST, an HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody (1:1,000) was applied for 1 h at 25°C in TBST containing 4% powdered milk (w/v). The membrane was washed with TBST, stained with enhanced chemiluminescence reagents, then imaged. Finally, the membrane was washed extensively with TBST and Coomassie Blue-stained to assess protein loading levels.

Peptide phosphorylation kinetics using a continuous colorimetric assay

Peptide phosphorylation was measured as described previously[1], using a colorimetric assay in which ADP production is coupled to NADH oxidation through two non-rate-limiting enzymatic steps, with concomitant loss of NADH absorbance at 340 nm. In these experiments, the reaction solutions contained 50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM phosphoenolpyruvate, 300 μg/mL NADH, 2 mM sodium orthovanadate, 100 μM ATP, and an excess of pyruvate kinase and lactate dehydrogenase. The ZAP-70 kinase domain was used at a concentration of 1 μM, and LAT peptides were used at a concentration of either 100 μM or 500 μM. Reaction components, except for the kinase, were mixed into the wells of a 96-well microplate, and the kinase was added from a concentrated stock to initiate the reaction. The reactions were carried out at 25°C, and NADH absorbance at 340 nm was measured every 10 sec. The relative reaction rates were inferred from the slopes of the initial linear phases of the reaction progress curves.

Isothermal calorimetry SH2 binding measurements

Binding affinities for the PLCγ1 N-terminal SH2 domain to two phosphopeptides were carried out by isothermal calorimetry using a MicroCal Auto iTC 200 calorimeter at 25°C. The peptides were, WT: ADEDEDD(pY)HNP**G**(pY)LVVLPDS and G131D: ADEDEDD(pY)HNP**D**(pY)LVVLPDS. In each case, the SH2 domain was placed in the cell at a concentration of 3 μM, and peptide was delivered in 16 injections via the syringe from a 30 μM stock solution. Each injection lasted 5.2 sec, during which 2.6 μL of peptide solution was delivered to the cell, and the injections were spaced by 7 min. All measurements were carried out at 25°C in a buffer containing 25 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mM TCEP. The number of binding sites (n) was not constrained during data fitting, and for all reactions, $0.8 > n > 1.1$, consistent with an expected 1:1 peptide:SH2 complex.

We note that previous measurements with LAT-derived peptides have clearly shown that PLCγ1 binds with at least 10-fold tighter affinity to a phospho-Y132 sequence, when compared to sequences bearing

a +2 Gln residue[3]. Thus, it is unlikely that this SH2 domain will bind significantly to the phospho-Y127 site on our peptides. We chose the doubly-phosphorylated peptide as this most likely resembles the cellular state of LAT when PLCγ1 binds, as Y127 should be phosphorylated faster than Y132[1]. We also note that our measured binding affinities are approximately 5-fold tighter than those reported previously[3]. This difference likely reflects a lack of phosphate ions in our buffers.

High-throughput peptide phosphorylation and binding screens

The activity of the ZAP-70 catalytic domain toward a library of all possible single point mutants in a peptide spanning LAT Y132 (residues 120-139 in a Y127F background) was determined previously using a high-throughput specificity-profiling platform[1]. A subset of these data is presented again in this manuscript for clarity. The previously-used screening platform combined bacterial surface display of genetically-encoded peptide libraries with fluorescence-activated cell sorting and deep sequencing of the peptide-coding genes. This platform was recently adapted to the analysis of SH2 domain sequence specificity[4]. We utilized the same procedure to measure the binding of a GFP-labeled tandem PLCγ1 N-SH2 construct to a phosphorylated form of the aforementioned LAT Y132 point mutant library. Briefly, after expression of the surface-displayed peptide library on *E. coli* MC1061 cells, the cells were phosphorylated to completion using a cocktail of 2.5 μM each of the ZAP-70, c-Abl, and c-Src kinase domains at 37°C for 1 h. Cells were washed with a neutral pH buffer, then incubated with the SH2-SH2-GFP construct (5 μM) on ice for 1 h. Cells with the highest GFP fluorescence intensity (top quartile) were isolated by cell sorting. The peptide-coding DNA from these sorted cells was deep sequenced and compared to that from unsorted cells. Enrichments of each peptide, reported on a $\log_{10}$-scale relative to the wild-type sequence, were calculated as described previously[1, 4].

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were isolated by cell sorting. The peptide-coding DNA from these sorted cells was deep sequenced and compared to that from unsorted cells. Enrichments of each peptide, reported on a $\log_{10}$-scale relative to the wild-type sequence, were calculated as described previously[1, 4].

Intracellular calcium measurements using a Flex Station

Jurkat variants stimulated with anti-CD3 and ionomycin.

To measure calcium mobilization using a Flex Station II (Molecular Probes), 1×10^7 Jurkat variants were washed with PBS twice, and loaded with 1 μM Indo-1 AM calcium indicator dye (Thermo Fisher Scientific) individually at 37 °C for 30 min in 2 ml RPMI medium. After loading with Indo-1, cells were washed with PBS twice, and resuspended at a concentration of 5×10^6 cells per ml. 5×10^5 cells (100 μl) were transferred into individual wells in a flat-bottom 96-well plate. The temperature in Flex Station II was set to 37 °C and each plate was incubated in the Flex Station II for 5 min before the experiment was started. Changes in the fluorescence ratio (violet/blue) were recorded every 4 sec. The cells were first left unstimulated for 30 sec to record basal levels of fluorescence intensities, followed by the addition of anti-CD3 (clone OKT3, Weiss lab) at the 30th sec and the addition of ionomycin (Thermo Fisher Scientific) at a later time point as specified in corresponding figure legends. Data were imported into GraphPad Prism software for analysis and production of graphs.

Jurkat variants stimulated with OVA or APL-loaded biotinylated monomers.

Jurkat variants were loaded with the calcium indicator dye Indo-1 as stated above (1 μM Indo-1 at 37 °C for 30 min). After loading cells with Indo-1, cells were washed with PBS twice, and resuspended in HBSS supplemented with 2% FBS at a concentration of 5×10^6 cells per ml. Cells were then transferred to individual wells in a flat-bottom 96-well plate at the concentration of 5×10^5 cells per well and incubated with 1:100 dilution of OVA or APL-loaded biotinylated pMHC monomers (NIH Tetramer Core Facility) at 37 °C for 30 min in the pre-warmed Flex Station II. To perform the experiment, indo-1 fluorescence ratios were recorded for 30 sec to obtain the baseline relative calcium levels, followed by the addition of streptavidin (10 μg/ml; Jackson Immunoresearch) at the 30th sec and this was followed by the addition of ionomycin (Thermo Fisher Scientific) at the 240th sec. Calcium changes were recorded for an additional 2 min after this last addition. For temperature-dependent experiments, cells were loaded with Indo-1 and incubated with OVA or control biotinylated monomers as above. Then, the plate was moved to 25 °C for about 10 min. The experiment was initiated with the temperature of Flex Station II set to 25 °C. The experiment at 25 °C was recorded as above. Afterwards, the temperature of Flex Station II was set to 37 °C. After the temperature reached 37 °C and maintained through a few minutes, the experiment at 37 °C was performed and recorded as above. Data were imported into GraphPad Prism software for analysis and production of graphs.

Immunoblot analysis

Jurkat and derivative cells were washed with PBS and resuspended at 5×10^6 cells/ml and rested for 30 min at 37^{o}C. Cells were left unstimulated or stimulated with anti-CD3 (clone OKT3) over time as described in each experiment. Cells were lysed by directly adding 10% NP-40 lysis buffer to a final concentration of 1% NP40 (containing inhibitors of 2 mM NaVO4, 10 mM NaF, 5 mM EDTA, 2 mM PMSF, 10 μg/ml Aprotinin, 1 μg/ml Pepstatin and 1 μg/ml Leupeptin). Lysates were placed on ice and

centrifuged at 13,000 × *g* to pellet cell debris. Supernatants were run on NuPAGE 4%–12% Bis-Tris Protein Gels (Thermo Fisher Scientific) and transferred to PVDF membranes. Membranes were blocked using TBS-T buffer containing 3% BSA, and probed with primary antibodies as described in each experiment, overnight at 4 °C. The following day blots were rinsed and incubated with HRP-conjugated secondary antibodies (Jackson Immunoresearch). Blots were developed using a chemiluminescent substrate and a BioRad Chemi-Doc imaging system (Bio-Rad).

Reference:

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4. Cantor, A.J., Shah, N.H. & Kuriyan, J. Deep mutational analysis reveals functional trade-offs in the sequences of EGFR autophosphorylation sites. *Proc. Natl. Acad. Sci. U. S. A.* **115**, E7303-E7312 (2018).